

ORIGINAL ARTICLE

Pleistocene divergence across a mountain range and the influence of selection on mitogenome evolution in threatened Australian freshwater cod species

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Climatic differences across a taxon's range may be associated with specific bioenergetic demands and may result in genetics-based metabolic adaptation, particularly in aquatic ectothermic organisms that rely on heat exchange with the environment to regulate key physiological processes. Extending down the east coast of Australia, the Great Dividing Range (GDR) has a strong influence on climate and the evolutionary history of freshwater fish species. Despite the GDR acting as a strong contemporary barrier to fish movement, many species, and species with shared ancestries, are found on both sides of the GDR, indicative of historical dispersal events. We sequenced complete mitogenomes from the four extant species of the freshwater cod genus *Maccullochella*, two of which occur on the semi-arid, inland side of the GDR, and two on the mesic coastal side. We constructed a dated phylogeny and explored the relative influences of purifying and positive selection in the evolution of mitogenome divergence among species. Results supported mid- to late-Pleistocene divergence of *Maccullochella* across the GDR (220–710 thousand years ago), bringing forward previously reported dates. Against a background of pervasive purifying selection, we detected potentially functionally relevant fixed amino acid differences across the GDR. Although many amino acid differences between inland and coastal species may have become fixed under relaxed purifying selection in coastal environments rather than positive selection, there was evidence of episodic positive selection acting on specific codons in the Mary River coastal lineage, which has consistently experienced the warmest and least extreme climate in the genus.

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INTRODUCTION

Evolution by natural selection has a fundamental role in shaping the geographic distribution of lineages (Avice, 2000). Understanding the distribution of genetic diversity across landscapes and patterns of local adaptation among and within lineages is an important component of effective conservation management, informing captive breeding programmes, translocations, 'genetic rescue' and targeted application of conservation resources (Harrison *et al.*, 2014). Taxa that span heterogeneous landscapes and/or strong environmental gradients are commonly associated with strong patterns of genetic and/or phenotypic variation and are therefore good candidates for exploring local adaptation (Schoville *et al.*, 2012).

An obvious potential driver of local adaptation is climate, because climatic differences across a taxon's range may be associated with specific bioenergetic demands and involve metabolic adaptations (Franks and Hoffmann, 2012). Because ectothermic organisms in aquatic systems rely on heat exchange with the environment to regulate many key physiological, developmental and behavioural processes (including metabolism), fish are highly sensitive to climatic and thermal conditions (Narum *et al.*, 2013). Thermal regime is therefore considered a key determinant of fish species' distributions and acts as a strong selective agent (Caissie, 2006).

Extending down the east coast of Australia, the Great Dividing Range (GDR) is Australia's most significant mountain range (~3500 km in length, up to ~300 km in width and up to ~2228 m in height) and has had a key role in freshwater fish speciation in eastern Australia (Unmack, 2001) (Figure 1). The GDR has a strong influence on the eastern Australian climate, with topographic relief leading to a typically arid/semi-arid climate inland and more mesic conditions (mean annual precipitation: evaporation ratio of >0.4) along the coast (Byrne *et al.*, 2011). Despite the GDR acting as a strong barrier to fish movement, many species, and species with recent shared ancestries, are found on both sides of the GDR (Unmack, 2001; Faulks *et al.*, 2015). Because the GDR predates most freshwater fish radiations in Australia, faunal similarity across the range is thought to have occurred by dispersal across the range (for example, by river-capture events for species that diverged tens of millions of years ago, or by extreme floods in areas of low elevation for more recently diverged species) (Unmack, 2013; Faulks *et al.*, 2015).

Maccullochella is represented by four extant species of large, predatory freshwater cod in the Percichthyidae family: the Murray cod *M. peelii*, the trout cod *M. macquariensis*, the Mary River cod *M. mariensis* and the eastern freshwater cod *M. ikei*. Species of *Maccullochella* occur on both sides of the GDR. The Murray cod and trout cod are endemic to the Murray-Darling Basin on the inland side

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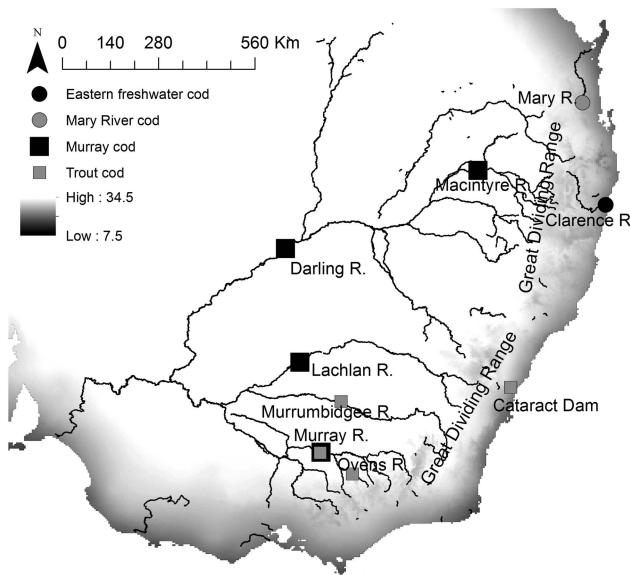


Figure 1 Sampling locations for sequenced *Maccullochella* individuals across South Eastern Australia. Major rivers and the GDR are shown. Grey shading reflects the mean annual temperature range (7.5–34.5 °C).

of the GDR and the eastern freshwater cod and Mary River cod persist in isolated coastal catchments to the east of the GDR (Figure 1) (Lintermans *et al.*, 2005). All *Maccullochella* cod species have suffered severe declines in population size though the twentieth century because of a range of anthropogenic pressures including habitat loss and degradation, altered flow and temperature patterns, in-stream sedimentation, pollution, introduced fishes, over-fishing and population fragmentation due to in-stream barriers (Lintermans *et al.*, 2005). The two coastal species, the Mary River and eastern freshwater cod, are each now restricted to single isolated drainage systems: the Mary River system in South Eastern Queensland and the Clarence River system in North Eastern New South Wales, respectively (Figure 1). Up until the early twentieth century, *Maccullochella* also occurred in intermediate drainages between the Clarence and Mary Rivers (predominantly in the Richmond and Brisbane Rivers) (Nock *et al.*, 2010). The inland Murray cod is widespread, but the trout cod is restricted to a handful of locations in the southern parts of the Murray-Darling Basin (Lintermans *et al.*, 2005). A stocked population of trout cod exists outside of its native range in Cataract Dam, east of the GDR (Figure 1).

Previous phylogenetic analyses suggest that the Mary River and eastern freshwater cod are sister taxa, and that the Murray cod is more closely related to the two coastal species than to the other inland congeneric, the trout cod (Nock *et al.*, 2010; Lavoué *et al.*, 2014). On the basis of data from two mitochondrial genes (ATPase 6 and ATPase 8), the two coastal species (eastern freshwater cod and Mary River cod) are thought to have diverged from each other around 300 (120–510) thousand years ago, following a crossing of the GDR by *Maccullochella* during the early- to mid-Pleistocene, around 1.09 (0.62–1.62) million years ago (Nock *et al.*, 2010). The number and directionality of historical crossings of the GDR by *Maccullochella* are not clear, but a single crossing in the direction of west to east has previously been hypothesised on the basis of the fossil record (Rowland, 1993; Nock *et al.*, 2010).

The mitochondrial genome, encoding 22 transfer RNAs (tRNAs), two ribosomal RNAs (rRNAs) and 13 structural proteins in

vertebrates, has proved a tractable system for exploring adaptive evolution, particularly evolutionary adaptations related to metabolic function (da Fonseca *et al.*, 2008). In coordination with the nuclear genome, the mitochondrial genome contributes substantially to metabolic performance, having a key role in energy and heat production through oxidative phosphorylation. There are five major protein complexes that contribute to the oxidative phosphorylation (OXPHOS) pathway in animals: complexes I, III, IV and V contain nuclear and mitochondrial encoded proteins, whereas complex II contains only nuclear encoded ones. Mitochondrial genes involved in the OXPHOS pathway can have important adaptive roles (for example, related to thermal adaptation and aerobic capacity) and be shaped by natural selection (da Fonseca *et al.*, 2008; Garvin *et al.*, 2014).

Because of its key role in energy production, the mitochondrial genome is under strong functional constraint (Meiklejohn *et al.*, 2007). However, despite a background of strong purifying selection, particular sites in the mitochondrial genome can experience positive selection in response to environmental pressures. Many studies have reported positive selection acting on mitochondrial protein-coding genes in vertebrates in response to environmental factors, including in mammals (da Fonseca *et al.*, 2008), birds (Stager *et al.*, 2014; Morales *et al.*, 2015) and fish (Silva *et al.*, 2014). Many of these studies find that candidate sites for positive selection are disproportionately concentrated in the NADH complex genes (OXPHOS complex I), which produces ~40% of the proton flux required for ATP synthesis (Garvin *et al.*, 2014).

In this study, we sequenced complete mitogenomes from the four extant species of *Maccullochella* and constructed a dated phylogeny to improve on previously reported divergence estimates based on two mitochondrial genes (Nock *et al.*, 2010). We also explored the relative influences of purifying and positive selection in the evolutionary history of mitogenome divergence among species. Different climatic conditions exist east and west of the GDR, with the inland environment characterised by a highly variable arid or semi-arid climate and the coastal environment characterised by more mesic conditions (Figure 1; Table 1). We hypothesised that divergence of the two coastal *Maccullochella* species would be associated with evolutionary change in response to a different climate. In particular, we explored whether there was evidence of positive selection acting on mitogenomes in the two coastal species, potentially as a result of adaptation to warmer and more consistent climate and thermal regimes east of the GDR.

METHODS

Sampling

Murray cod and trout cod samples were sourced from four and three different inland locations across the Murray-Darling Basin, respectively (Figure 1). Trout cod sampled from the Murrumbidgee and Ovens Rivers represent translocated fish originally of Murray River origin. Three additional trout cod samples were sourced from Cataract Dam, a coastal population outside the trout cod's natural range consisting of stocked fish (Figure 1). The translocations occurred too recently on evolutionary timescales (early twentieth century) for novel adaptive mutations to be likely to have occurred in Cataract Dam, and thus samples from Cataract Dam are classified as inland samples for the purposes of analyses. The specific interest of sampling these trout cod is that the dam was stocked in around 1916 during the 'Nature's Waste' programme that sought to salvage fish impacted by severe drought: trout cod were translocated from the Murrumbidgee Catchment, where the species is now naturally extinct (Trueman, 2011). The two coastal Mary River cod samples were sourced from the Mary River and the three coastal eastern freshwater cod samples came from the Clarence River (Figure 1). Additional collection details for samples are

Table 1 Geographic coordinates for sampling locations and associated WORLDCLIM (Hijmans *et al.*, 2005) climate data: maximum temperature of warmest month (°C), minimum temperature of coldest month (°C), mean annual precipitation (mm) and mean annual temperature range (°C)

Region	Location	Latitude	Longitude	Max temp warmest month (°C)	Min temp coldest month (°C)	Precipitation (mm)	Annual temp range (°C)
Coastal	Clarence River	-29.49632 ^a	153.29391 ^a	27.2	8.3	36	18.9
Coastal	Mary River	-26.83406 ^a	152.69057 ^a	27.7	5.6	45	22.0
Coastal	Cataract Dam	-34.26566	150.81000	25.5	3.9	28	21.7
Inland	Murrumbidgee River	-34.64000	146.37400	32.3	3.4	13	28.9
Inland	Darling River	-30.64600	144.92600	35.2	5.0	27	30.2
Inland	Lachlan River	-33.61600	145.30000	32.7	3.6	15	29.1
Inland	Murray River	-35.96628	145.85256	31.2	3.4	17	27.8
Inland	Macintyre River	-28.60233	149.96233	33.8	4.5	33	29.3
Inland	Ovens River	-36.53400	146.66933	28.7	2.0	29	26.7
Inland	Murray River	-35.98070	145.82780	31.2	3.4	16	27.8

Climate variables are derived from monthly temperature and rainfall data.

^aGeographic coordinates for the Clarence River and Mary River samples are approximate sampling coordinates as exact GIS locations were unavailable.

provided in Supplementary material (Supplementary Table S1). The complete mitogenome sequence of the nightfish *Bostockia porosa* (sister group to *Maccullochella*; Lavoué *et al.*, 2014) (Genbank accession AP014529) was used as an outgroup in phylogenetic analyses.

Climatic differences across the GDR

Different climatic conditions exist east and west of the GDR. The Mary River and Clarence River systems are classified as humid subtropical (Peel *et al.*, 2007). In contrast, the majority of the Murray-Darling Basin is considered arid or semi-arid and is characterised by a highly variable climate (Peel *et al.*, 2007). Climate data were downloaded from WorldClim (Hijmans *et al.*, 2005) and visualised in ArcGIS 10.2. Relative to inland locations, coastal locations were associated with narrower annual temperature ranges, lower maximum temperatures in the hottest month, higher minimum temperatures in the coldest month and higher annual precipitation (Figure 1; Table 1).

Mitogenome sequencing and annotations

Genomic DNA was extracted from fin clips using the Qiagen Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Purified DNA was physically fragmented to 800 bp using a Covaris220 (Covaris, Woburn, MA, USA) and prepped using the NEXTflex DNA-seq library preparation kit (Bioo Scientific, Houston, TX, USA). The libraries were quantified, normalised, pooled and sequenced on the MiSeq Desktop Sequencer (2 × 250 bp read-length configuration) located at the Monash University Malaysia Genomics Facility. The raw paired-end reads were interleaved and assembled using the MITObim V 1.6 wrapper script (Hahn *et al.*, 2013), which relies on the MIRA 3.4.1.1 assembler for mitochondrial baiting and iterative mapping (Chevreux *et al.*, 1999). The consensus sequence for each assembled mitogenome was defined using the standard MIRA algorithm (with default settings), which accepts or refuses reads on the basis of quality score, number of reads, presence of forward and reverse reads, and any potential inexplicable errors introduced into the consensus (see Supplementary Data 1 in Wilson *et al.*, 2012 for further details). The assembled mitogenomes were subsequently annotated using MITOannotator (<http://mitofish.aori.u-tokyo.ac.jp/>) (Iwasaki *et al.*, 2013). As crude quality control for potential presence of nuclear pseudogene in the assembly, each mitogenome annotated by MITOannotator was inspected for missing genes and abnormal gene length (because of the presence of early stop codons). Mitogenomes were aligned and manually checked using GENEIOUS 6.1.3 (<http://www.geneious.com/>). Annotated genomes were submitted to Genbank (accession numbers: KT337324-KT337338).

Genetic diversity and divergence

The number of haplotypes (H), haplotype diversity (Hd) and nucleotide diversity (π) were calculated in DnaSP v.5 (Librado and Rozas, 2009) for each

of the 13 protein-coding genes, tRNAs and rRNAs in each species. For each of the protein-coding genes, the number of fixed synonymous and non-synonymous mutations between the species was calculated in DnaSP. Between-lineage p-distances were calculated in MEGA 6.06 (Tamura *et al.*, 2011). Consistent with Nock *et al.* (2010), we were unable to align the control region (CR) of trout cod with the other three species and so the CR was removed from all analyses.

Phylogenetic analysis

Bayesian phylogenetic analysis (MrBayes) was performed on the concatenated alignment of the 13 protein-coding genes to generate the phylogeny required for input into subsequent codon-based tests for selection. Gaps, intergenic spacers and stop codons were removed from the alignment and overlapping regions between ATPase 8 and ATPase 6 and between ND4L and ND4 were resolved by repeating the overlapping bases to ensure each gene was fully represented and to prevent disruption to the reading frame. ND6 is encoded on the light strand, so for consistency, the reverse-complemented sequence was used in phylogenetic reconstruction. We identified the optimal partitioning scheme, and the optimal models of molecular evolution for each subset partition, using the Bayesian Information Criterion (BIC) as implemented in PartitionFinder (Lanfear *et al.*, 2012) for both the protein-coding dataset and complete mitogenomes (excluding control region). Bayesian phylogenetic reconstruction analysis was then conducted in MrBayes 3.2 (Ronquist *et al.*, 2012). The analysis was run for 10 million generations sampled every 1000 generations with four independent runs to assess convergence. Run convergence was checked using TRACER 1.6.0 (Rambaut and Drummond, 2007). Trees were summarised using the 50% majority rule method after discarding the first 25% of the sample as burnin and visualised using FIGTREE 1.4.2 (Rambaut, 2007). The MrBayes consensus tree with the outgroup removed was used in codon-based selection analyses described below (results were robust to inclusion/exclusion of outgroup). We also inferred amino acid sequences at the ancestral node preceding the split between trout cod and the other three species using the method implemented in CODEML, under the phylogeny obtained from the analysis described above.

Divergence time estimates

We estimated divergence times between *Maccullochella* cod species using BEAST 1.8.2 (Drummond *et al.*, 2012). Analyses were run using complete mitogenomes (excluding the control region) and the nine partitions determined using PartitionFinder (Supplementary Table S3), with unlinked substitution and clock models but linked tree topologies and applying the Yule process (appropriate for species divergence estimates) starting with a random tree. Because the Yule process assumes each operational taxonomic unit is a different species, inclusion of multiple individuals per species may affect estimates of divergence dates. We therefore ran the BEAST analysis including only one

individual per species. Because inclusion of codons under positive selection may bias divergence estimates, we also removed putatively selected codons from the analysed dataset. Clock rates were chosen to encompass a range of mitochondrial time-dependent rates for comparably recent (<3 million years) divergences of galaxiids and other fishes, calibrated on the basis of geologically dated changes in river drainages and isolation events (0.003–0.13 substitutions/site/million years; Burrige *et al.*, 2008). To account for uncertainty in clock rates, we sampled clock rates from a distribution rather than setting fixed rates. We assigned partitions either a 'slow' or 'fast' clock rate distribution on the basis of typical patterns of rate variation among positions. The clock rate was sampled from a log-normal distribution with mean (in real time)=0.01 substitutions/site/million years, log standard deviation=0.8 and starting value=0.01 for partitions 1–4, 6 and 8 ('slow' clock; 1st and 2nd codon positions, tRNAs and rRNAs; Supplementary Table S3), and mean=0.04, log sd=0.8 and starting value=0.04 for partitions 5 and 7 ('fast' clock; 3rd codon positions; Supplementary Table S3). The prior distribution was truncated to a maximum of 0.5 substitutions/site/million years (that is, the pedigree rate of control region in humans; Santos *et al.*, 2005). The 'slow' and 'fast' clock priors corresponded to 95% of molecular clock rates being sampled between 0.002 and 0.04 substitutions/site/million years and between 0.006 and 0.14 substitutions/site/million years, respectively. To check for possible rate heterogeneity among lineages, we initially ran the analysis using an uncorrelated log-normal relaxed clock model. The initial analysis was run for 100 million generations and sampled every 10 000 steps. Because the *ucl.dstdev* parameter estimates for all partitions included zero and/or were not greater than one, rate heterogeneity among branches was rejected and the final analysis was run using a strict clock model (Supplementary Table S4). For the final analysis, five replicate runs were checked for convergence, combined and analysed in Tracer 1.6.0 after discarding the first 10% of samples in each run as burnin.

Codon-based approaches to detecting positive selection associated with divergence across the GDR

Estimating codon-based ω ratios. The ratio of non-synonymous to synonymous substitutions ($\omega = dN/dS$) is a measure widely used to detect selection, with $\omega > 1$ indicative of positive selection, $\omega < 1$ indicative of purifying selection and $\omega = 1$ indicative of neutrality (Yang, 2007). Because strong purifying selection acts to preserve mitochondrial function and can mask positive selection acting on individual sites, we used phylogeny-based models implemented as described below, to estimate $\omega = dN/dS$ for each codon rather than for entire genes/genomes (Meiklejohn *et al.*, 2007; da Fonseca *et al.*, 2008).

We used CODEML implemented in PAML4 (Yang, 2007) to run a series of maximum likelihood models and subsequently made model comparisons designed to test for signatures of positive selection associated with particular codons. First, we ran the recommended six M-series site models (M0, M1a, M2a, M7, M8; Table 2) to examine whether there was selection acting on particular codons independent of phylogenetic branch. We compared M0 (one ω ratio) with M1a (nearly neutral; two classes ω ratios: $\omega_0 < 1$ and $\omega_1 = 1$) to test for evidence of non-neutrality, and compared M1a with M2a (adds a third class of ω ratios to M1a: $\omega > 1$) and M7 (beta) with M8 (beta and ω) to test for

evidence of positive selection (Yang, 2007) (Table 2). Second, because different lineages may have experienced different selection regimes (for example, inland vs coastal species) we ran the branch model (Model 2). We compared the two- ω branch model (allows different ω for coastal and inland) with M0 as a test for different ω values on the coastal and inland branches. To complement the branch model comparisons, we also used GA-branch model selection implemented in the web-server Datamonkey (Delpont *et al.*, 2010). In contrast to CODEML models, GA-branch does not require *a priori* selection of branches of interest, but instead uses a model selection approach to partition and estimate ω values across the tree. Third, we ran branch-site-model-A-modified in CODEML to test for episodic selection affecting particular sites in particular lineages. In branch-site-model-A-modified, background lineages (defined as inland here) are restricted to ω values ≤ 1 and the focal foreground branches (defined as coastal here) are allowed ω values > 1 . A comparison of branch-site-model-A-modified with M1a tests for positive selection in the foreground (coastal) lineages, however, this comparison is unable to distinguish between positive selection and relaxation of purifying selection (Zhang *et al.*, 2005). The branch-site-model-A-modified was thus compared with the null model (in which ω is fixed at 1) as a more stringent test of positive selection as outlined in Zhang *et al.* (2005). All model comparisons were made using a likelihood ratio test.

In addition to CODEML models, we used the programme HyPhy implemented in the web-server Datamonkey (Delpont *et al.*, 2010) to identify codons putatively under selection using Fast Unconstrained Bayesian Approximation (FUBAR; Murrell *et al.*, 2013) and Mixed Effects Model of Evolution (MEME; Murrell *et al.*, 2012). FUBAR assigns each codon a posterior probability (PP) of belonging to three classes of ω : $\omega_0 < 1$, $\omega_1 = 1$ and $\omega_2 > 1$. Codons with PP > 0.9 and $\omega > 1$ or $\omega < 1$ were inferred to have evolved under positive and purifying selection, respectively. Whilst FUBAR identifies codons putatively under selection across all branches on the phylogeny, MEME allows ω at each codon to vary across branches/lineages and so detects episodic selection (that is, positive selection that varies temporally across branches/lineages). Codons with $P < 0.05$ were considered to have experienced episodic positive selection in certain lineages.

Detecting radical changes in physicochemical properties of amino acids. Because ω ratios > 1 may not reflect molecular adaptation *per se*, we used TREESAAP (Woolley *et al.*, 2003) to look for significant changes in physicochemical properties associated with amino acid replacements in inferred proteins on particular branches in the phylogeny. TREESAAP uses a sliding window approach to compare observed patterns of amino acid replacements and changes in physicochemical properties to those expected under neutrality (Woolley *et al.*, 2003). Amino acid replacements that have strong effects on protein biochemistry are considered candidates for selection (Woolley *et al.*, 2003). We ran the analysis with a sliding window size of 20 codons, increasing in 1-codon increments. To minimise false positives, only amino acid replacements with magnitude categories ≥ 6 and z-scores above/below ± 3.09 ($P < 0.001$) were considered as potentially under positive/purifying selection, respectively (McClellan and Ellison, 2010). We only considered amino acid

Table 2 CODEML model comparisons and associated hypothesis that is being tested are described

Model comparison	Log ratio test	Testing for
Model 0 vs Model 1a	$\chi^2 = 40.04$, $P < 0.05$	Non-neutrality
Model 1a vs Model 2a	$\chi^2 = 0$, NS	Positive selection
Model M7 vs Model M8	$\chi^2 = 1.43$, NS	Positive selection
Model 0 vs branch model 2	$\chi^2 = 20.55$, $P < 0.001$	Higher ω ratios in pre-specified foreground branches (coastal branches here)
MAnull vs branch-sites-model-A-modified	$\chi^2 = 2327.9$, $P < 0.001$	Positive selection or relaxation of selective constraint affecting certain sites in pre-specified foreground branches (coastal branches here)
Model 1a vs branch-sites-model-A-modified	$\chi^2 = 18.99$, $P < 0.001$	Positive selection (if $\omega_2a > 1$ and/or $\omega_2b > 1$ in foreground), or relaxation of selective constraint (if ω_2a and/or $\omega_2b \leq 1$ in foreground) affecting certain sites in pre-specified foreground branches (coastal branches here)

Bold font indicates a significant ($P < 0.05$) log ratio test and support for the stated hypothesis.

properties with an accuracy of detecting selection >85% (thus, excluding 11 out of the 31 properties tested) (McClellan and Ellison, 2010).

RESULTS

Per-base coverage varied across samples (mean per base coverage = 121; range = 25–336) and complete mitogenome sequence lengths varied among species: eastern freshwater cod and Mary River cod: 16 437 bp, Murray cod: 16 442 bp and trout cod: 16 479 bp (Supplementary Table S2). Two of the eastern freshwater cod samples (EC002 and EC806) had identical mitogenome sequences (including the CR), so EC806 was removed from analyses. We detected no gene-order rearrangements in any lineage. All *Maccullochella* cod species had an additional amino acid towards the 3'-end of ND1 and two additional amino acids in ND5, relative to the outgroup sequence *B. porosa*. Conversely, *B. porosa* had an additional amino acid at the 3'-end of ND5 and two additional amino acids at the 3'-end of Cytb. The *B. porosa* outgroup mitogenome sequence was >25% divergent from the *Maccullochella* cod species.

Genetic diversity and divergence

Within-species nucleotide diversity (π) was low for all the *Maccullochella* cod species: Murray cod = 0.0004, trout cod = 0.0003, Mary River cod = 0.0003, eastern freshwater cod = 0.0001 (Supplementary Figure S1). The majority of within-species diversity was located in protein-coding genes for all species, with no variation at any of the tRNAs or rRNAs in the two coastal species (Supplementary Figure S1).

Mean uncorrected p-distances for the complete mitochondrial genomes (excluding control region) between species were: 12.0% for Murray cod vs trout cod, 1.1% for Murray cod vs Mary River cod, 1.2% for Murray cod vs eastern freshwater cod and 0.04% for Mary River cod vs eastern freshwater cod. There were 13 fixed non-synonymous differences between the coastal (Mary River cod and eastern freshwater cod) and inland (Murray cod and trout cod) lineages (Table 3). In 11 of the 13 instances of non-synonymous fixed differences between inland and coastal species, the same amino acid was maintained in both inland species (Table 3). In addition, there were fixed differences at 12 nucleotide sites in the rRNAs and tRNAs:

12S rRNA ($N=1$), 16S rRNA ($N=7$), tRNA-Val ($N=2$), tRNA Met ($N=1$) and tRNA-Arg ($N=1$) (Supplementary Figure S2).

Phylogenetic analysis

PartitionFinder supported 9 partitions for the complete mitochondrial genome sequences (excluding control region), which were used in the BEAST analysis. Eight partitions were supported for the protein-coding dataset, which were subsequently used for phylogeny estimation (the same phylogeny was supported for the complete mitochondrial sequences, but we present only results from the protein-coding dataset because downstream codon-based tests for selection were based on protein-coding sequence) (Supplementary Table S3). All partitions were considered informative for differences between the three closely related cod species (Murray cod, Mary River cod and eastern freshwater cod; Supplementary Table S3). All four cod species were monophyletic and well supported (PP = 1.0; Figure 2).

Divergence time estimates

Posterior estimates of substitution rates ranged from 0.02 (95% HPD 0.01–0.03) substitutions per site per million years for partition 4 (2nd codon positions) to 0.04 (0.02–0.07) substitutions per site per million years for partition 9 (1st codon position for ND6; Supplementary Tables S3 and S4). On the basis of complete mitochondrial genomes excluding control region and the 11 putatively positively selected sites, the mean estimate of time to the most recent common ancestor of all four *Maccullochella* species was 6.17 (3.15–9.62) million years. The most recent common ancestor of Murray cod and the coastal cod species (Mary River cod and eastern freshwater cod) was 450 (220–710) thousand years and the most recent common ancestor of Mary River cod and eastern freshwater cod was 140 (68–230) thousand years (Figure 2; Supplementary Table S3).

Detecting positive selection associated with divergence across the GDR

Variation in codon-based ω ratios. CODEML site models found support for non-neutrality (model M1a), but no evidence of positive selection (models 2a or 8) (Table 2 and Supplementary Table S6).

Table 3 Fixed amino acid differences between coastal (eastern freshwater cod and Mary River cod) and inland (Murray cod and trout cod) *Maccullochella* species

OXPHOS complex	Codon	Gene	Ancestral state	Inland		Coastal		TREESAP Significant properties ($P < 0.001$, categories 6–8)
				Murray	Trout	Mary River	Eastern freshwater	
I	252	ND1	S	S	S	L	L	$\uparrow R_F$
I	62	ND2	V	V	V	I	I	$\downarrow \rho K'$
I	243	ND2	L	L	L	F	F	—
I	293	ND2	V	V	V	I	I	$\downarrow \rho K'$
V	14	ATPase 6	F	F	F	L	L	—
V	48	ATPase 6	S	S	S	N	N	—
IV	40	COIII	M	M	M	T	T	$\downarrow P_\alpha$
IV	168	COIII	V	V	I	A	A	—
I	108	ND3	T	T	T	M	M	$\downarrow P_\alpha$
I	68	ND4	T	T	T	I	I	—
I	357	ND4	A	A	A	T	T	$\downarrow P_\alpha$
I	395	ND4	T	T	T	A	A	$\downarrow P_\alpha$
I	448	ND5	H	S	H	F	F	—

Standard amino acid codes are given for each species and the inferred ancestral state (reconstructed using CODEML). Codon number refers to codon number in gene. Bold font indicates properties with evidence of positive selection based on global Z scores. TREESAP codes for properties are: R_F = Chromatographic index, $\rho K'$ = Equilibrium Constant – ionisation, COOH, $P_\alpha = \alpha$ – helical tendencies. Increase or decrease in amino acid properties from inland to coast is indicated with up and down arrows.

Under the supported non-neutral model (M1a), 97% of codons (p_0) were estimated as being under purifying selection independent of phylogenetic branch (with the remaining 3% behaving neutrally) (Supplementary Table S6). Model selection in GA-Branch supported

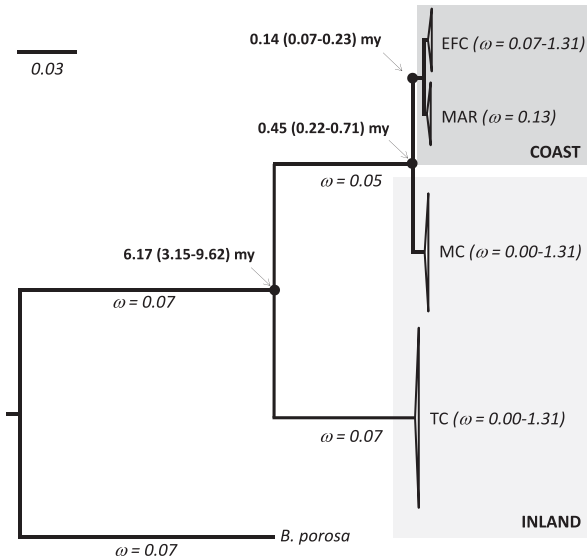


Figure 2 Rooted phylogeny of the *Maccullochella* genus based on 13 protein-coding mitochondrial gene sequences, estimated using Bayesian inference in MrBayes. All nodes were well supported (PP=1.0). BEAST 95% HPD estimates for time to most recent common ancestor (in million years) for nodes indicated by black circles are given. Ratios of non-synonymous to synonymous substitutions (ω values) inferred from the branch partitioning scheme chosen through GA-branch model selection are indicated in italics below each branch and in parentheses following species labels for species branches and tips. Very short branches within-species clades have been collapsed for better presentation; triangle height corresponds to number of sequences and triangle width corresponds to diversity within clade. Species codes are: Murray cod (MC, $N=4$), trout cod (TC, $N=6$), Mary River cod (MAR, $N=2$) and eastern freshwater cod (EC, $N=2$). The outgroup is the nightfish *Bostockia porosa*.

five different ω values along the tree (Figure 2). Although the majority of inland and coastal branches had $\omega < 1$, indicative of purifying selection, the coastal branches were generally associated with higher ω values than the inland branches (Figure 2). Higher ω ratios associated with coastal species compared with inland species was also supported by both CODEML branch and branch-site models. CODEML branch models estimated higher ω ratios in coastal species ($\omega=0.16$) compared with inland species ($\omega=0.05$) (model 2; Table 2 and Supplementary Table S6). The branch-site model found support for variable ω across codons and branches, with higher ω ratios associated with 13.5% of sites in foreground (coastal) branches compared with background (inland) branches (branch-sites-model-A-modified; Table 2 and Supplementary Table S6). Because the estimated values of ω_{2a} and ω_{2b} were < 1 in the foreground branches, support for the branch-site-model-A-modified was consistent with relaxation of purifying selection in the foreground (coastal) branches rather than positive selection (Table 2 and Supplementary Table S6).

FUBAR supported strong, pervasive purifying selection acting across all mitochondrial protein-coding genes independent of phylogenetic branch, finding 79.6% of codons showed evidence of purifying selection at posterior probability ≥ 0.9 and no codons with evidence of pervasive diversifying selection at posterior probability ≥ 0.9 (results not shown).

MEME found evidence of episodic diversifying selection at 11 codons ($P < 0.05$) (Table 4). Seven of these 11 codons were associated with the coastal lineages, 6 of these 7 with divergence of the Mary River cod (Table 4). Four of the seven sites showing evidence of episodic diversifying selection in the coastal lineages were located in OXPHOS complex I and three in OXPHOS complex IV (Table 4).

Radical changes in the physicochemical properties of amino acids. On the basis of global Z scores, nine of the physicochemical properties tested in TREESAAP showed overall patterns of purifying selection across the phylogenetic tree (Supplementary Table S7). Only two of the properties tested showed an overall pattern of positive selection (alpha-helical tendencies and equilibrium constant (ionisation of COOH); Supplementary Table S7 and Figure 3). Specific codons

Table 4 Codons with evidence of episodic positive selection detected by MEME

OXPHOS complex	Codon	Gene	Ancestral state	Inland		Coastal		Sample/species (location) affected	MEME P-value	TREESAAP Significant properties ($P < 0.001$, categories 6–8)
				Murray	Trout	Mary River	Eastern freshwater			
I	261	ND1	T	T	T	T	T/A ^a	EC002 (Clarence R.)	0.013	$\uparrow P_{\alpha}$
I	314	ND1	P	P	P	Q	P	MAR (Mary R.)	0.030	—
IV	88	COI	G	G/V ^a	G	G	G	MCC0105 (Darling R.)	0.009	$\downarrow P$
IV	366	COI	V	V	V	I	V	MAR (Mary R.)	0.046	$\downarrow pK'$
IV	21	COII	I	I	I	I/L ^a	I	MAR001 (Mary R.)	0.046	$\uparrow pK'$
IV	107	COIII	A	A	A	T	A	MAR (Mary R.)	0.047	$\downarrow P_{\alpha}$
I	37	ND5	A	A/D ^a	A	A	A	MCC0105 (Darling R.)	0.008	$\downarrow E_t$
I	357	ND5	N	N	N/S	N	N	TC_B5 (Ovens R.)	0.013	—
I	528	ND5	H	H	H	Y	H	MAR (Mary R.)	0.049	—
III	80	Cytb	R	R/Q ^a	R	R	R	MCC0105 (Darling R.)	0.013	—
I	170	ND6	L	L	L	I	L	MAR (Mary R.)	0.026	$\downarrow pK'$

Abbreviation: MEME, Mixed Effects Model of Evolution. Codon number refers to codon number in gene. OXPHOS complex and gene for each codon, the amino acids present in each of the four cod species and the sample ID/species associated with the amino acid replacement are given. Standard amino acid codes are given for each species and the inferred ancestral state (reconstructed using CODEML). Species codes in sample IDs are: Murray cod (MCC), trout cod (TC), Mary River cod (MAR) and eastern freshwater cod (EC).

MEME P -values are given for codons identified as under episodic positive selection. Significant ($P < 0.001$, categories 6–8) TREESAAP properties associated with the amino acid replacements are given, and the directions of change are indicated. Bold font indicates properties with evidence of positive selection based on global Z scores. TREESAAP codes for properties are: P = Turn tendencies, E_t = Total non-bonded energy, pK' = Equilibrium Constant – ionisation, COOH, P_{α} = α – helical tendencies. Amino acid replacements affecting coastal lineages are shaded.

^aIndicates only one individual has amino acid replacement.

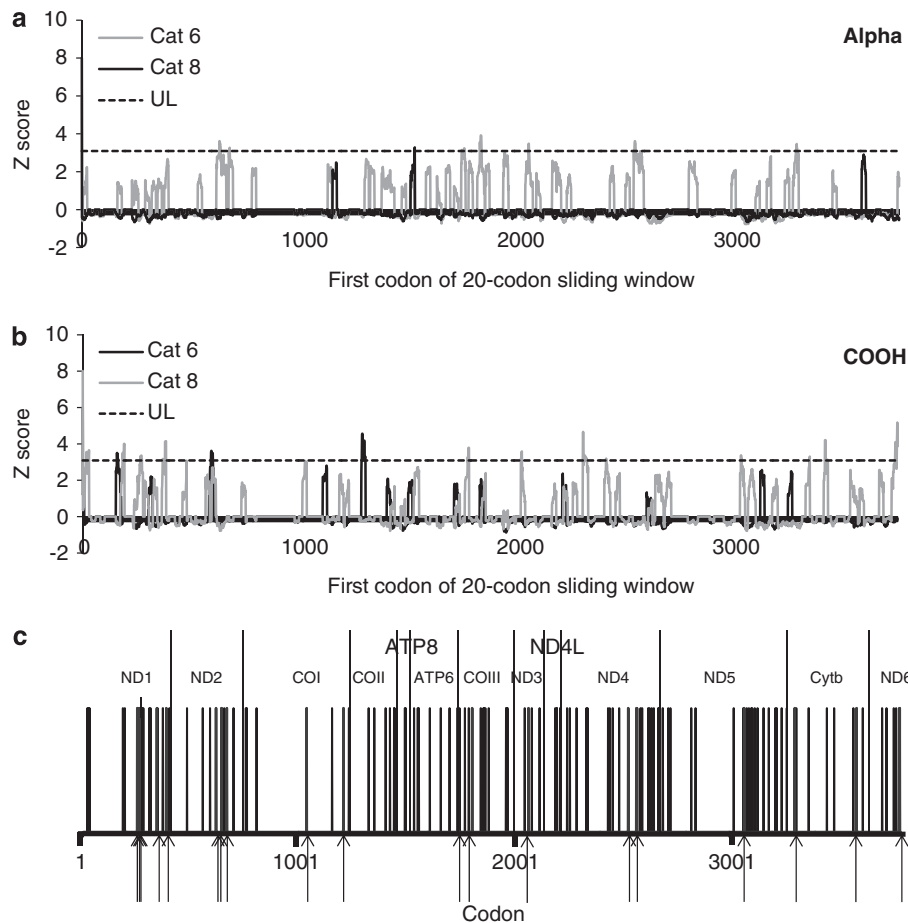


Figure 3 Evidence of significant changes in physicochemical properties of amino acids detected using TREESAAP analysis. For categories 6 and 8, Z scores greater than 3.09 (above the dotted line) indicate overall significant radical changes in the physicochemical properties: (a) alpha-helical tendencies and (b) equilibrium constant (ionisation of COOH). The x axis for (a) and (b) represents a sliding window (20 codons) increasing in one-codon implements. Codons associated with significant radical amino acid changes on particular branches of the tree are represented by black lines in (c). Arrows below x axis indicate significant radical amino acid changes associated with the coastal branches of the tree. The x axis in (c) represents a specific codon position.

associated with significant ($P < 0.001$) radical (categories 6–8) changes in amino acid properties were detected across all the mitochondrial protein-coding genes (Figure 3). The majority of radical changes detected were associated with divergence of the trout cod (73.7% or 98/133). Divergence between the Murray cod and the ancestor of two coastal species was associated with 14.3% (19/133) of the radical changes detected (Figure 3). Seven of the 13 amino acid differences fixed between the inland and coastal species were associated with significant radical changes in physicochemical properties (Table 3). Seven of the 11 codons detected as showing evidence of episodic positive selection by MEME were also associated with significant radical changes in physicochemical properties in TREESAAP analysis (Table 4). As a proportion of gene length, OXPHOS complex I had a slightly higher proportion of codons showing evidence of significant radical changes in physicochemical properties (Figure 4).

DISCUSSION

Phylogenetic analyses supported mid- to late-Pleistocene divergence of *Maccullochella* across the GDR (220–710 thousand years ago) and were consistent with the revised taxonomy based on shorter mitochondrial DNA fragments published by Nock *et al.* (2010), with the two coastal species being sister taxa and, in turn, a sister group to Murray cod (Figure 2). We detected overall strong, pervasive purifying selection

acting across the mitogenome in *Maccullochella* species, as has been observed across many other taxa (Meiklejohn *et al.*, 2007). Although population diversity metrics based on small sample sizes should be interpreted with caution, very strong purifying selection was consistent with very low mitogenome diversity within species, even in the widely distributed Murray cod, for which only six synonymous and four non-synonymous changes were detected across our broadly distributed samples (Figure 1). Although purifying selection appeared to dominate the evolution of the mitochondrial genome in *Maccullochella*, the strength of selection (ω values) did vary among sites and among branches on the tree and there was evidence of potentially functionally relevant fixed amino acid replacements between the inland and coastal species. Although many of the amino acid differences between inland and coastal species may have been the result of relaxed purifying selection rather than positive selection, we did detect some evidence of episodic positive selection acting on specific codons in specific coastal lineages, particularly associated with divergence of the Mary River cod.

Mid- to late-Pleistocene divergence of *Maccullochella* across the GDR

Analysis of complete mitochondrial genomes allowed us to generate more precise estimates of divergence times among *Maccullochella* than those obtained previously by Nock *et al.* (2010), even while

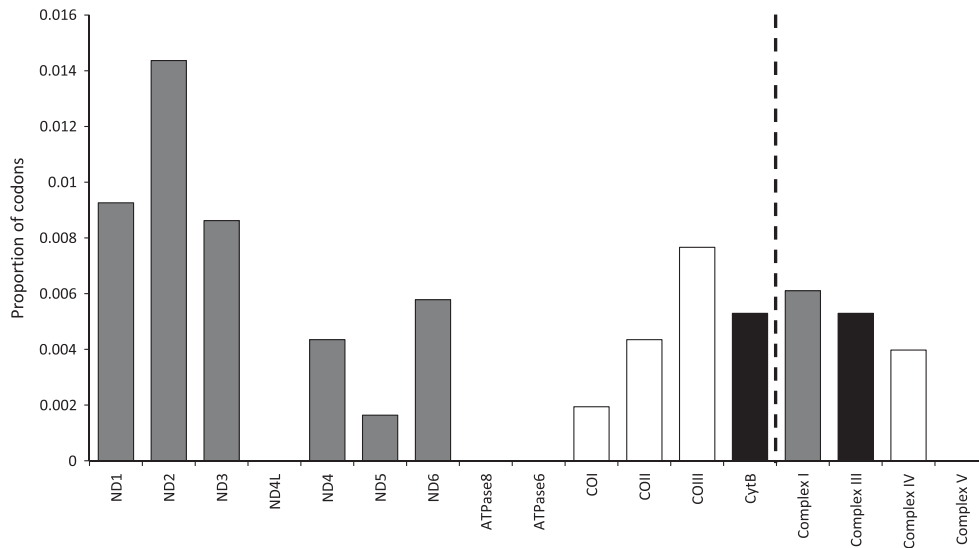


Figure 4 Proportion of codons relative to gene length in each mitochondrial protein-coding gene/complex (complexes are colour-coded) that show evidence of significant positive disruptive selection in coastal *Maccullochella* lineages in TREESAAP analysis.

incorporating uncertainty in evolutionary rates. Phylogenetic analysis suggests that divergence between the inland and coastal *Maccullochella* species occurred 450 (220–710) thousand years ago during the mid- to late-Pleistocene, bringing forward the bounds of previously reported dates based on two mitochondrial genes (0.62–1.62 million years; Nock *et al.*, 2010). Similarly, our data suggested more recent divergence between the two coastal species (~140 (68–230) thousand years ago) than previously estimated (120–510 thousand years; Nock *et al.*, 2010). Comparable estimates of mitogenome divergence across the GDR were obtained for another endemic freshwater fish species, the Macquarie Perch *Macquaria australasica*, suggesting that the same geological/environmental conditions may have facilitated GDR crossing for multiple species (119–385 thousand years; Pavlova *et al.*, unpublished data). During the Pleistocene, cycles of alternating warm, moist interglacial climate conditions and cool, dry glacial climate conditions shaped fish distributions by influencing the amount of freshwater habitat and connectivity among drainages (Faulks *et al.*, 2015). Movement across the GDR by *Maccullochella* was most likely facilitated by extreme flooding in low elevation regions of the GDR (Nock *et al.*, 2010; Unmack, 2013). Drainage isolation as a result of increasingly dry conditions through the Pleistocene and/or increased sea levels during interglacial periods may have led to divergence between the Mary River and eastern freshwater cod east of the GDR (Byrne *et al.*, 2011; Faulks *et al.*, 2015).

Although we excluded sites showing evidence of positive selection from the BEAST analysis, strong, pervasive purifying selection acting across the mitogenome detected in our study does violate the molecular clock's assumption of neutrality. However, because pervasive purifying selection is widespread among taxa, it is expected that similar selective processes would have occurred on a comparable timescale in the evolution of the taxa on which we based our clock rate priors (Meiklejohn *et al.*, 2007; Burrige *et al.*, 2008).

Potential relaxation of purifying selection in coastal species may be driven by relaxed metabolic constraints and/or historical population bottlenecks

Relaxed purifying selection in both coastal species was supported by higher ratios of non-synonymous to synonymous changes (ω values) associated with coastal branches in both CODEML and GA-Branch

analyses. Reduced selective constraint on OXPHOS mitochondrial genes could be the result of relaxed metabolic constraints in the milder climate east of the GDR and/or reduced effectiveness of selection owing to reductions in effective population size associated with historical population bottlenecks (Hughes, 2007). In addition, lack of strong evidence of positive selection using the CODEML method may reflect low power because of relatively recent divergence of the coastal species and associated low polymorphism (Melo-Ferreira *et al.*, 2014).

Environments and lifestyles with high energy demands (for example, extreme climate, flight ability, migratory behaviour) are typically associated with stronger functional constraint on metabolism (that is, lower ω values associated with codons/genes linked to energy metabolism) (Melo-Ferreira *et al.*, 2014). Given the climatic differences across the GDR, the coastal environment may be associated with a relaxation of selective constraint on genes linked to tolerance of temperature extremes and/or variability. However, historical population bottlenecks (for example, associated with colonisation of coastal environments by *Maccullochella*) and small effective population size can also lead to reduced efficiency of selection and the accumulation of mildly deleterious changes. Identifying whether relaxed metabolic constraint or reduced efficiency of selection drives signatures of relaxed selection is challenging, especially as the two processes are not mutually exclusive. For example, higher ratios of non-synonymous to synonymous changes in mitogenomes of a number of domesticated (*cf.* wild) taxa have been attributed to both relaxed selective constraint on metabolic efficiency because of human actions (for example, provision of resources and improved living conditions) and reduced efficiency of selection owing to repeated population bottlenecks during the domestication process (Hughes, 2013). In our case, rigorously distinguishing among metabolic adaptation to coastal environments (positive selection), small effective population in coastal environments (reduced efficiency of selection) and reduced metabolic constraint in coastal environments (relaxed selection) would be impossible without controlled experiments and/or studies of protein biochemistry. For example, better metabolic efficiency of inland than coastal species under more variable temperature regimes would support relaxed selection in the coastal environment as the driving process. Alternatively, greater metabolic efficiency of coastal than inland species

under less variable temperature regimes would support positive selection acting on coastal species.

Some evidence of functional differences across the GDR and episodic positive selection in coastal lineages, particularly associated with divergence of the Mary River cod

Although CODEML analyses did not support positive selection acting consistently on mitogenomes across coastal lineages, there was evidence of potentially functionally relevant fixed amino acid differences across the GDR and of possible episodic positive selection acting on specific codons in specific coastal lineages (TREESAAP and MEME analysis), particularly associated with the divergence of the Mary River cod. In the case of the Mary River cod, signatures of potential positive selection acting on the mitogenome have accumulated after a relatively short period of evolutionary time (68–230 thousand years).

Of the 13 non-synonymous fixed differences (that is, amino acids not shared) between inland and coastal species, 11 were represented by the same amino acid in both inland species. Given the strong levels of mitochondrial divergence (~12%) observed between the two inland species (Murray cod and trout cod), conservation of the same amino acid in Murray cod and trout cod for differences that were fixed across the GDR could reflect strong selective pressures for these amino acids in environments west of the GDR that are relaxed or different east of the GDR. Seven of the 13 amino acid differences fixed between the inland and coastal species were associated with significant changes in physicochemical properties (six of these changes relating to properties with evidence of positive selection based on global Z scores) suggesting that functional differences exist between mitogenomes east and west of the GDR that may have consequences for metabolic function (Woolley *et al.*, 2003).

Six of the eleven sites identified by MEME as being putatively under episodic positive selection were associated with the Mary River cod (the more northern of the two coastal species), with five of these being fixed amino acid differences between the Mary River cod and all other *Maccullochella* species and mostly associated with radical changes in physicochemical properties of amino acids. Amino acid replacements putatively under selection in the Mary River cod were located in complexes I (ND1, ND5 and ND6) and, somewhat atypically, in complex IV (COI, COII and COIII) (Zhang and Broughton, 2013). Whilst amino acid replacements in complex I (ND genes) tend to have minor effects on functional properties of amino acids, amino acid replacements in complex IV (COX genes) tend to have disproportionate effects on fitness, making COX genes typically the most highly conserved in the mitogenome (Zhang and Broughton, 2013). Although sites showing signatures of episodic positive selection detected in the Mary River cod may represent false positives that are in fact deleterious but not (yet) removed by selection because of low effective population size, investigation of candidate sites and adaptive processes in Mary River cod seems warranted (Hughes, 2007).

Concluding remarks

We report mid- to late-Pleistocene divergence of *Maccullochella* across the GDR (220–710 thousand years ago), followed by relatively recent divergence of the two coastal species (68–230 thousand years ago). We detected potentially functionally relevant fixed amino acid differences between the inland and coastal species of *Maccullochella*. Although many of the amino acid differences between inland and coastal species may have been the result of relaxed purifying selection rather than positive selection, there was evidence of episodic positive selection acting on specific codons in specific coastal lineages, particularly the Mary River cod, which experiences relatively stable, and the warmest,

environments in the genus. Our study establishes a foundation for future experimental investigations into adaptive processes in species of *Maccullochella*, including the functional effects of candidate amino acid replacements on metabolism.

DATA ARCHIVING

Sequence data have been submitted to GenBank: accession numbers: KT337324-KT337338.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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